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Research report

Cortisol responses to chronic stress in adult macaques: Moderation by a polymorphism in the serotonin transporter gene

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HIGHLIGHTS

- Rh5-HTTLPR exerted an influence on cortisol responses to stress in macaques.
- The *s*/*s* genotype was associated with increased cortisol responses to stress.
- In the absence of stress, no differences related to genotype were observed.
- This moderation was a genetic modulation of cortisol responses to stress.

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ABSTRACT

Accumulating evidence has shown that a polymorphism in the promoter region of the serotonin transporter gene (5-HTTLPR) moderates the association between stress and depressive symptoms. However, the exact etiologies underlying this moderation are not well understood. Here it is reported that among adult female rhesus macaques, an orthologous polymorphism (rh5-HTTLPR) exerted an influence on cortisol responses to chronic stress. It was found that females with two copies of the short allele were associated with increased cortisol responses to chronic stress in comparison to their counterparts who have one or two copies of the long allele. In the absence of stress, no differences related to genotype were observed in these females. This genetic moderation was found without a genetic influence on exposure to stressful situations. Rather it was found to be a genetic modulation of cortisol responses to chronic stress. These findings indicate that the rh5-HTTLPR polymorphism is closely related to hypothalamus–pituitary–adrenal (HPA) axis reactivity, which may increase susceptibility to depression in females with low serotonin transporter efficiency and a history of stress.

1. Introduction

Mood disorders and major depressive disorder (MDD) are associated with disturbances of both the hypothalamus–pituitary–adrenal (HPA) axis [1] and the serotonergic system [2–4]. Anti-depressants, which are routinely prescribed for many depressed patients, serve to regulate the function of the HPA axis [5] and/or the serotonergic system [6].

The serotonin transporter (5-HTT), which transports serotonin (5-HT) back into the cell after its neurochemical message has been delivered, has been recognized to be a key regulator of serotonergic neurotransmission. The 5-HTT gene contains a 44 base pair deletion/insertion polymorphism in the promoter region (5-HTTLPR) and has received considerable attention as a regulatory mechanism by many researchers. This polymorphism in the 5-HTT gene consists of two functionally distinct promoter lengths; coined the short (*s*) and long (*l*) alleles, respectively. The short allele is

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associated with lower transcriptional efficiency and thus has lower 5-HTT availability than the long allele [7].

Caspi and colleagues reported that individuals with one or two copies of the short allele exhibited more depressive symptoms, diagnosable depression, and suicidality in relation to stressful life events (SLEs) than individuals homozygous for the long allele [8]. Since then, the 5-HTTLPR has been the focus of intensive research, although some researchers who applied meta-analytic techniques to data from relevant published studies failed to replicate this interaction [9,10]. Nonetheless, a more recent meta-analysis including all relevant studies has since found strong evidence that 5-HTTLPR moderates the relationship between stress and depression [11]. Yet, the mechanisms underlying this moderation are still not well understood.

Findings from animal and human researches suggest that genetic moderation of HPA axis reactivity to stressors may be one possible mechanism [12–14]. Murphy et al. found that mice with diminished (5-HTT+/−, heterozygote mice) or absent (5-HTT−/−, homozygote knockout mice) 5-HTT function exhibited more fearful behaviors and displayed greater increases in stress hormone response to stress when compared to homozygous (5-HTT+/+) controls [15]. In the absence of stress, no differences related to genotype were observed in these animals [15]. Research in humans also supported that HPA axis reactivity moderated by a polymorphism in the serotonin transporter gene is involved in the development of depression. Individuals with two short alleles at the 5-HTT locus were more susceptible to the depressogenic effects of SLEs than those with one or two long alleles [16]. In addition, Miller et al. used a meta-analysis to further evaluate this HPA axis reactivity in humans, and found that individuals homozygous for the short allele displayed increased cortisol reactivity to acute psychosocial stress compared with individuals with one or two copies of the long allele [17]. This "stress sensitivity" hypothesis was further verified by the finding that individuals homozygous for the short allele exhibited greater amygdala activation in response to fearful stimuli than individuals who have at least one long allele [18,19]. In turn, elevated amygdala reactivity leads to a hyperactivity of HPA-axis and an exaggerated stress response [20].

While most research into the "stress sensitivity" hypothesis was focused on the relationship between acute SLEs and the 5-HTTLPR [12–14], research into the effects of chronic stress and the 5-HTTLPR has been more challenging due to the variable nature of human societal systems [21]. Non-human primates (NHP) afford an opportunity to study the associations between the 5-HTTLPR, chronic stress and HPA axis reactivity because of their genetic similarity to humans, and more importantly, because their rearing environments can be tightly controlled [22,23]. For example, the rhesus macaque polymorphism (rh5-HTTLPR) was found to affect HPA-axis reactivity in infant macaques and that this genetic influence on hormonal responses during stress was modulated by early life experience [24]. The study presented here was designed to measure adult macaques' cortisol responses to chronic stress generated in long-term dominant–subordinate relationships and aimed to demonstrate that HPA-axis reactivity depends on both: the rh5-HTTLPR genotype and chronic stress in an adult sample.

2. Methods

2.1. Animals

Twenty-nine female rhesus macaques (*Macaca mulatta*) living in the Kunming Primate Research Center of the Chinese Academy of Sciences were selected at random and observed in this study. The animals came from 13 breeding groups and ranged from 11 to 24 years of age (15.03 ± 3.51 years). They lived in colonies with access to a connected indoor ($2.61 \times 2.46 \times 2.58$ m)–outdoor ($2.67 \times 2.66 \times 2.67$ m) cage. All animals were given commercial monkey biscuits twice a day with tap water *ad libitum*, and were fed with fruits and vegetables once daily. The animals had lived in their respective social groups for at least 1 year prior to initial observation.

All animal procedures were approved by the National Animal Research Authority (P.R. China) and the Institutional Animal Care and Use Committee (IACUC) of Kunming Institute of Zoology, Chinese Academy of Sciences. All efforts were made to minimize the monkeys' suffering. For example, hair samples were taken from the back of the monkey's neck using an electric-razor without anesthetic and no animals were sacrificed in this study. Routine veterinary care was provided throughout the study by professional keepers and veterinarians.

2.2. Experimental design

Animal behaviors in social hierarchies were video recorded using a focal follow technique [25] and were analyzed to calculate the chronic stress that they experienced based on dyadic interactions. After completion of the video recordings, blood and hair samples were obtained to measure the rh5-HTTLPR genotype and cortisol levels, respectively. Then, associations among the rh5-HTTLPR, chronic stress and hair cortisol levels were studied.

2.3. Behavioral sampling

The monkeys were given seven days to acquaint themselves with the observers and cameras prior to recording and sampling. Then, a digital camera fixed on a tripod was set up in front of the colony to record one monkey at a time in the cage. The observers kept as far away as possible (at least 5 m) from the monkeys' cages in order to avoid disturbing the animals during video recordings. Fourteen 1-h recordings were collected for each monkey on separate days sequentially throughout a six month period. All video recordings were stored on a hard disk and interpreted by three technicians. The three viewers analyzed each video recording simultaneously using a standardized behavioral classification [26]. The inter-rater reliability was scored in the SPSS software package (SPSS Inc., Chicago, IL, USA), which found the interclass correlation coefficient (ICC) to be >0.99. Each technician was blind to the genotype and cortisol levels of the animals.

2.4. Chronic stress

Chronic stress were quantized as conflict behaviors, which included aggressive and submissive behaviors. Aggressive behaviors included a bite, slap, grab, stare threat, open-mouth threat, chase, and a forced displacement. Submissive behaviors included a scream, scream threat, crouching, fleeing, lip smack, grimace, submissive present, and moving away [26]. Each of the above behaviors was scored as initiating (displaying) or receiving from another female and the frequencies of each behavior were calculated per hour.

2.5. Genotyping

DNA was isolated using standard extraction methods from whole blood collected from the femoral vein under ketamine anesthesia (15 mg/kg, i.m.) [27]. Genotyping was performed as described in previous studies [7,24,27]. The rh5-HTTLPR was amplified from 25 ng of genomic DNA with flanking oligonucleotide primers (stpr5, 5′-GGCGTTGCCGCTCTGAATACC; intl, 5′-CAGGGGAGATCCTGGGAGGGA). Amplicons were separated by electrophoresis on a 2% agarose gel. The short and long alleles of the

rh5-HTTLPR were identified by direct visualization after ethidium bromide staining, and were further confirmed by gene sequencing (performed by BGI, Shenzhen, China); with the short allele lacking a 22 bp sequence (GCCCTTTCAGCATCCCCCTGCA).

2.6. Hair sampling and cortisol extraction

Before commencing the experiment, the hair on the back of each animal's neck was shaved with an electric-razor without anesthetics, with particular attention made by technicians not to break or damage the skin. After six months, the video-recordings were completed and the new grown hair was collected as mentioned above. The hair samples were then placed into small pouches of aluminum foil and stored as previously described [28,29].

The hair cortisol extraction was performed as previously described [28,30]. The cortisol concentration in each sample was quantified with a radioimmunoassay (RIA) kit (Cortisol RIA DSL-2000, Texas, USA). The cortisol RIA was performed in triplicate under a double blind design at the Radioimmuno Laboratory of the Second Affiliated Hospital of the Kunming Medical College.

2.7. Data analysis

Data analysis was conducted using the SPSS software package (SPSS Inc., Chicago, IL, USA). A Chi-square test was used to verify whether this population was in accordance with the Hardy–Weinberg law. The normality of the data was computed using Kolmogorov–Smirnov test. In cases where data were not normally distributed, standard transformation procedures were used to achieve normality (natural log for conflict behaviors). A multi-variables linear regression was used to assess the main effects of rh5-HTTLPR genotype (with a 0 score used for those with one copy or two copies of the long allele and a score of 1 for *s*/*s* homozygotes), conflict behaviors and their interaction on HPA axis output (hair cortisol levels) among female macaques. In order to evaluate the influences of genotypes on the numbers of conflict behaviors that females experienced, a one-way ANOVA was conducted. In all analyses, the *p*-values were determined from two-sided tests and the significance level was set at $p < 0.05$.

3. Results

3.1. Genotype

Animals were divided into two groups based on their rh5-HTTLPR genotype: (1) those with two copies of the short allele (*s*/*s* homozygotes; $n = 7$; 24.1%) and (2) those with one copy (*s*/*l* heterozygotes; $n = 15$; 51.7%) or two copies of the long allele (*l*/*l* homozygotes; $n = 7$; 24.1%). The population was in Hardy–Weinberg equilibrium ($X^2 = 0.034$, $p = 0.853$).

3.2. The associations among rh5-HTTLPR, LEs and hair cortisol

Conflict events were quantified by the numbers of dyadic behaviors that each female experienced and were divided into four categories: (1) display of aggression, (2) display of submission, (3) receipt of aggression, and (4) receipt of submission. However, not all of these categories represent the chronic stress that the monkeys experience. For instance, the display of aggression and/or receipt of submission may have represented a stress-attenuating coping response, rather than a stress source [31]. Therefore, it is the receipt of aggression and/or the display of submission that represents the chronic stress experienced by the monkeys in dyadic interactions. The receipt of aggression is an external stressor and the display of submission represents an internal coping process [32].

A multi-variables linear regression was conducted to test the associations between hair cortisol and (i) rh5-HTTLPR genotype, (ii) receipt of aggression, and (iii) their interactive effect. It was found that the main effects of genotype ($b = 0.172$, $SE = 0.251$, $t = 0.684$, $p = 0.500$) and receipt of aggression were not significant ($b = 0.218$, $SE = 0.173$, $t = 1.263$, $p = 0.218$), but the interactive effect was significant ($b = 0.755$, $SE = 0.322$, $t = 2.344$, $p = 0.027$) on hair cortisol levels (Fig. 1A). The same analysis was then used to examine the role of display of submission. The main effects of display of submission ($b = 0.066$, $SE = 0.121$, $t = 0.544$, $p = 0.591$) and genotype ($b = 0.209$, $SE = 0.255$, $t = 0.819$, $p = 0.420$) were not significant, and their interactive effect was significant ($b = 0.657$, $SE = 0.268$, $t = 2.454$, $p = 0.021$) on hair cortisol levels (Fig. 1B). Next, aggression displays and receipt of submission, the two categories unlikely to be a source of stress, were analyzed for their effects on cortisol levels (Fig. 1C and D). Neither a main effect nor an interactive effect was found to be significant for either of these criteria.

3.3. Influences of genotypes on the numbers of experienced conflict behaviors

Genotypes did not have an influence on the frequencies of experienced conflict behaviors, including receipt of aggression ($p = 0.486$), display of submission ($p = 0.833$), display of aggression ($p = 0.122$) or receipt of submission ($p = 0.307$) (Fig. 2).

4. Discussion

Here, the first NHP study about the genetic basis of serotonin effects on adult HPA axis reactivity to chronic stress is presented. Dyadic behaviors between female macaques, a natural facet of monkey social structure, were used as indicators of chronic stress in this study. This study focused primarily on females because the effect of the serotonin transporter genotype (rh5-HTTLPR) on HPA axis reactivity has been shown to be sexually dichotomous in both humans and macaques. Female s-carriers have greater HPA axis responses to stressors than males of the same genotype [13,33,34]. An interactive effect between the rh5-HTTLPR genotype and chronic stress was found, in which *s*/*s* homozygotes secreted higher levels of cortisol than monkeys with the *s*/*l* and *l*/*l* genotype. This finding is consistent with a few human studies [13,14,35]. The effect was found not to be caused by genetic influences on the exposure to stressful situations. Rather, it was a genetic moderation of cortisol responses to chronic stress.

Extensive research has suggested that chronic stress can precipitate depression [36,37]. However, the underlying biological mechanism of this relationship is unknown. A broad literature base suggests that the serotonergic system, commonly perturbed in MDD, influences the stress-activated HPA axis response to trigger depressive episodes [12,38]. The role of the 5-HTT has been of particular interest as a polymorphism in the promoter region of the gene (5-HTTLPR) has been found to be associated with HPA axis responses in both humans and macaques [13,14,33,34]. In the present study, female macaques with two copies of the short allele displayed greater increases in the cortisol response to stress when compared to *l*-allele carriers. In the absence of stress, no differences related to genotype were observed in these females. This supports the "stress sensitivity" hypothesis that the short allele interacts with stress to push the HPA axis into dysfunction, which may contribute to the pathology of mood disorders [12].

Previous studies on the human serotonin transporter polymorphism and HPA axis reactivity have focused only on cortisol responses to acute stress [12–14], and neglected the role of chronic stress. The agonistic behaviors utilized in this study provided a stable, chronic stressor as they are common and long-standing

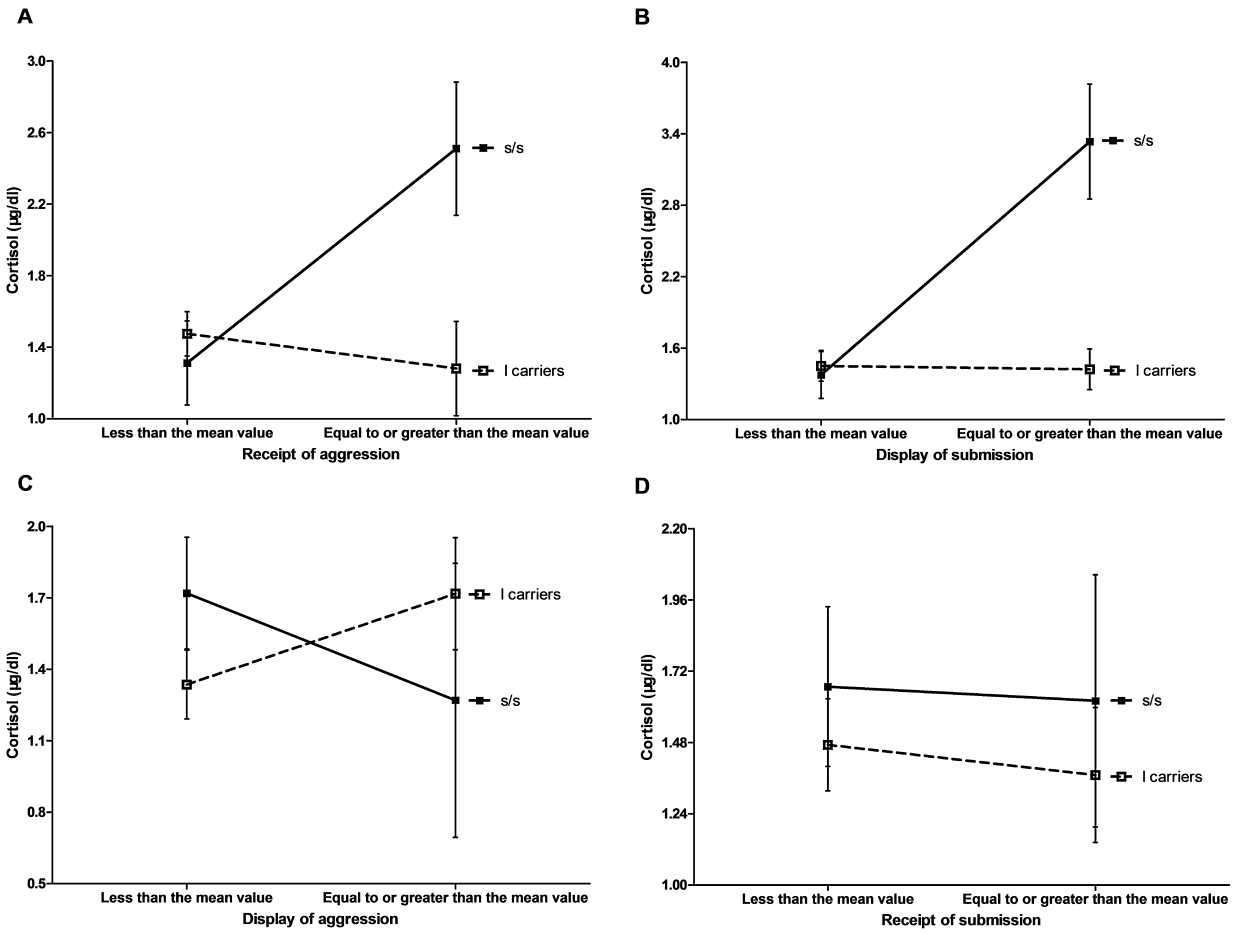

Fig. 1. Interactive effects of the rh5-HTTLPR genotype (*l* carriers or *s*/*s*) and conflict behaviors (less than the mean value or equal to or greater than the mean value) on cortisol responses (values listed are mean levels of cortisol in μg/dl ± SEM). Conflict behaviors were recorded in the form of receipt of aggression (A), display of submission (B), display of aggression (C) and receipt of submission (D). (A) Receipt of aggression. The main effect of rh5-HTTLPR was not significant ($b = 0.172$, $SE = 0.251$, $t = 0.684$, $p = 0.500$), the main effect of receipt of aggression was not significant ($b = 0.218$, $SE = 0.173$, $t = 1.263$, $p = 0.218$), and the interaction between rh5-HTTLPR and receipt of aggression was significant ($b = 0.755$, $SE = 0.322$, $t = 2.344$, $p = 0.027$). (B) Display of submission. The main effect of rh5-HTTLPR was not significant ($b = 0.209$, $SE = 0.255$, $t = 0.819$, $p = 0.420$), the main effect of display of submission was not significant ($b = 0.066$, $SE = 0.121$, $t = 0.544$, $p = 0.591$), and the interaction between rh5-HTTLPR and display of submission was significant ($b = 0.657$, $SE = 0.268$, $t = 2.454$, $p = 0.021$). (C) Display of aggression. The main effect of rh5-HTTLPR was not significant ($b = 0.279$, $SE = 0.265$, $t = 1.052$, $p = 0.302$), the main effect of display of aggression was not significant ($b = 0.153$, $SE = 0.186$, $t = 0.824$, $p = 0.418$), and the interaction between rh5-HTTLPR and display of aggression was not significant ($b = 0.859$, $SE = 0.783$, $t = -1.907$, $p = 0.283$). (D) Receipt of submission. The main effect of rh5-HTTLPR was not significant ($b = 0.181$, $SE = 0.260$, $t = 0.697$, $p = 0.492$), the main effect of receipt of submission was not significant ($b = -0.092$, $SE = 0.139$, $t = -0.667$, $p = 0.511$), and the interaction between rh5-HTTLPR and receipt of submission was not significant ($b = -0.142$, $SE = 0.378$, $t = -0.376$, $p = 0.710$).

within the monkey social structure [39]. Moreover, cortisol levels measured in previous studies only reflected short-term stress occurring over hours to days and did not assess chronic stress levels occurring over weeks to months without repeated samplings [40,41]. Hair has a fairly predictable growth rate of approximately 1 cm/month. Hence, the most proximal 1 cm segment to the scalp approximates the last month's cortisol production, the second most proximal 1 cm segment approximates the cortisol production during the month before that and so on [29]. Furthermore, cortisol levels in hair samples are not affected by the stress of the sampling procedure [42,43]. All told, this makes cortisol measured in hair a strong biomarker of chronic stress, which has been shown in two previous rhesus macaque studies [28,30]. In this experiment, the cortisol levels were measured from hair grown during the behavioral sampling period, suggesting it represented the mean cortisol production associated with the chronic stress the animals experienced.

Another challenge in human stress studies is the inability to control for complex factors that affect human environments. For example, studies that examine stressful life events as an environmental factor often do not account for the quality and availability of social support, which are a potent buffer for stress [44]. However, a recent study has proposed that specific environments contribute influence on gene–environment interactions when they are related to interpersonal events, rather than non-interpersonal ones [35], which is consistent with the monkey study presented here as the animals have lived in a closely related social hierarchy. Nonetheless, the conceptualization and measurement of stressful life events in humans has been highly variable across previous studies [21]. Some researchers have argued that there only exists a loose relationship between environmental stressors and subjective evaluation of their stressfulness [45], which critically affects gene–environment interactions.

Relatively speaking, studies in NHP models rely on quantitative, readily observable measures of stress that allow for a well-controlled design in which environmental factors can be manipulated. Therefore, the gene–environment interactions can be dissected in a controlled but ethologically relevant context in rhesus macaques by separately measuring the genetic, stress and cortisol components in the animals. Although the results presented here represent a small sample size, the data in adult female macaques adds credence to the "stress sensitivity" model of the

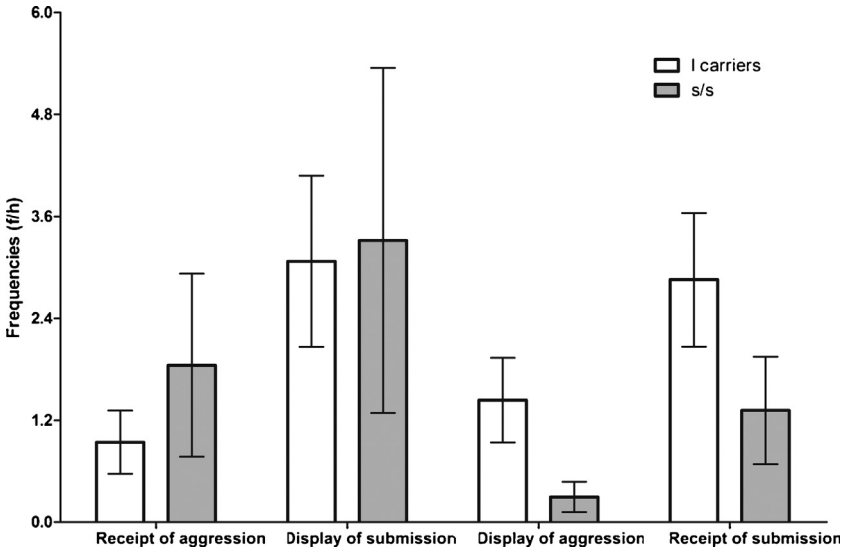

Fig. 2. Influences of rh5-HTTLPR genotype (*l* carriers or *s*/*s*) on the frequencies of conflict behaviors that monkeys experienced (values listed are mean frequencies per hour (f/h) ± SEM). Genotypes did not affect the frequencies of experienced conflict behaviors, including receipt of aggression ($p = 0.486$), display of submission ($p = 0.833$), display of aggression ($p = 0.122$) or receipt of submission ($p = 0.307$).

5-HTT polymorphism in depression etiology. As such, replication of these findings in a larger population will allow further development of similar NHP models to pursue physiological experiments probing the neural and neuroendocrine influences of the serotonin transporter gene polymorphism. These models will provide a foundation for future studies to identify biological substrates related to the variable responses to chronic stress.

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